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## Correlation of plasma osteoprotegerin (OPG) and receptor activator of the nuclear factor $\kappa$ B ligand (RANKL) levels with clinical risk factors in patients with advanced carotid atherosclerosis

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
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### Summary

#### Background:

Osteoprotegerin (OPG) is considered to be a crucial regulatory mediator of bone metabolism by acting as a decoy receptor of the receptor activator of nuclear factor  $\kappa$ B ligand (RANKL). OPG and RANKL have further become the subject of intense interest for their potential role in cardiovascular disease. The present study aimed to assess the clinical implication of plasma OPG and RANKL levels in patients with advanced carotid atherosclerosis

#### Material/Methods:

Plasma OPG and RANKL concentrations measured by solid-phase enzyme-linked immunosorbent assay (ELISA) were correlated with medical history, risk factors and medication intake in 131 patients who underwent carotid endarterectomy for vascular repair.

#### Results:

Plasma OPG concentrations were associated with patients' age ( $p=0.0258$ ), homocysteine levels ( $p<0.00001$ ), eGFR ( $p=0.0254$ ), history of diabetes ( $p=0.0324$ ), statins therapy ( $p=0.0044$ ), hyperlipidemia ( $p=0.0407$ ), smoking ( $p=0.0226$ ) and CAD ( $p=0.0377$ ). Plasma RANKL concentrations were associated with patients' age ( $p=0.0191$ ), homocysteine levels ( $p<0.00001$ ), history of smoking ( $p=0.0185$ ) and statins therapy ( $p=0.0004$ ). Diabetes, CAD, smoking status, statins therapy and homocysteine were identified as independent predictors of OPG concentrations ( $p=0.0157$ ,  $p=0.0030$ ,  $p=0.0249$ ,  $p=0.0047$  and  $p=0.0072$ , respectively), whereas smoking showed an independent effect for RANKL ( $p=0.0010$ ).

#### Conclusions:

The present data reinforce the clinical utility of OPG in carotid atherosclerosis, whereas the clinical implication of RANKL seems uncertain.

#### key words:

**OPG • RANKL • atherosclerosis • carotid • medical history • risk factors**

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## BACKGROUND

Osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) receptor superfamily, which was originally discovered as an inhibitor of osteoclastogenesis [1]. OPG is a soluble glycoprotein consisting of 380 amino acids, which exists in 2 forms – as a monomeric form of 60 kDa and as homodimeric form linked with disulfide bond of 120 kDa, which is the active form [2]. It is widely expressed in most human tissues, including osteoblasts of the bone, as well as in endothelial and smooth muscle cells of the vascular wall [3]. OPG acts as a soluble decoy substrate to the receptor activator of the nuclear factor  $\kappa$ B ligand (RANKL). RANKL, a transmembrane glycoprotein of the TNF superfamily, is expressed by osteoblasts, stromal cells and T lymphocytes and binds to RANK, which is located to the surface of osteoclast precursor cells such as monocytes, macrophages and dendritic cells [4,5]. RANKL-RANK interactions activate nuclear factor  $\kappa$  by degradation of I $\kappa$ B protein by I $\kappa$ B kinase, leading to release of the nuclear factor  $\kappa$ B (NF $\kappa$ B), which then translocates to the nucleus in order to initiate the transcription of specific genes required for osteoclast differentiation [6,7]. Binding of OPG to RANKL competitively attenuates RANKL-RANK interactions and inhibits the proliferation and differentiation of osteoclasts and consequently bone resorption [8]. Bone constitutes the largest source of OPG and RANKL molecules. Notably, the OPG/RANKL/RANK axis exerts pleiotropic effects on bone metabolism and hormonal secretion, being considered responsible for ossification and bone mineralization [8]. Moreover, OPG is able to bind TNF-related apoptosis-inducing ligand (TRAIL), which is expressed by T lymphocytes, endothelial and smooth muscle cells and exerts protective effects by preventing apoptotic cell death of infiltrating inflammatory cells during atherosclerosis and cardiovascular disease [9,10].

Atherosclerosis constitutes a chronic inflammatory disease occurring within the artery wall and is a main cause of cardiovascular diseases such as myocardial infarction and stroke [11]. Stress, tobacco smoking, alcohol consumption, hypertension, diabetes mellitus, obesity and dyslipidemia have been identified as important risk factors predisposing to atherosclerosis [12]. Atherosclerotic plaque formation is triggered by endothelial cell activation and dysfunction causing the release of vasoactive molecules, which stimulate an inflammatory response and recruitment/migration of leukocytes into the intima of the arterial wall [13]. The ensuing secretion of cytokines, growth factors and mediators promote vascular smooth muscle cell proliferation and potentiate the inflammatory response associated with arterial remodelling [14,15]. The multi-factorial nature of atherosclerosis involves chronic inflammation at every step from initiation to progression, suggesting that certain clinical risk factors may contribute to the pathogenesis of disease by aggravating the underlying inflammatory process [16].

The strong association between bone pathologies and atherosclerosis has stimulated systematic research for the identification of common molecular mediators linking the skeletal and the vascular systems [17,18]. In this aspect, several bone turnover regulators and structural proteins, including OPG and RANKL, were shown to be expressed within atherosclerotic plaques [19]. Substantial data from animal models have recently suggested that OPG may exert a

protective role against pathological calcification within the vascularity, being a potential marker of the onset of atherosclerosis [20–25]. Notably, several clinical studies have indicated a strong association between OPG elevation and cardiovascular disease states, including coronary artery disease (CAD), peripheral artery disease (PAD), acute myocardial infarction, heart failure, abdominal aortic aneurism, vascular calcification and stroke [26–29]. However, there has been little clinical evaluation of circulating OPG and RANKL in advanced carotid atherosclerosis. Interestingly, it was shown that early and advanced human carotid atherosclerotic lesions presented elevated OPG and RANKL immunoreactivity and mRNA expression [30,31]. Moreover, circulating and tissue OPG elevation was associated with carotid plaque echogenicity and neurological symptomatology [30,32]. Another study based on a healthy population documented an inverse relationship between serum OPG levels and carotid plaque echogenicity [33]. Serum OPG levels were also positively associated with carotid intima thickness in women with gestational diabetes [34]. In view of the above considerations, the present study aimed to assess the plasma OPG and RANKL concentrations in patients with advanced carotid atherosclerosis in relation to medical history, risk factors and medication intake.

## MATERIAL AND METHODS

### Patients

The study enrolled 131 patients that underwent carotid endarterectomy in Laikon Hospital between January 2007 and December 2008. The study was approved by the Hospital Ethics Committee. Informed consent was obtained from all participants. Indication for surgery was a symptomatic carotid stenosis of  $\geq 50\%$  or an asymptomatic carotid stenosis of  $\geq 70\%$  [35]. Patients preoperatively had carotid duplex ultrasound scans, digital subtraction angiograms, or both. Patients or plaques were defined as symptomatic when focal symptoms of cerebral ischemia were present, ipsilateral to the carotid lesions, such as transient ischemic attack, amaurosis fugax, or stroke occurred in the last 6 months. All patients were on antiplatelet treatment preoperatively, which was interrupted 1 week before surgery.

A complete medical history, risk factors and medication intake were recorded, including age, sex, coronary artery disease (CAD) (angina pectoris, myocardial infarction and coronary artery by-pass grafting/percutaneous transluminal coronary angioplasty-CABG/PTCA), diabetes mellitus (controlled with diet, oral hypoglycemic agents or insulin; fasting glucose level  $\geq 126$  mg/dL), hyperlipidemia (total cholesterol  $\geq 200$  mg/dL), hypertension (systolic blood pressure  $\geq 140$  mmHg, diastolic blood pressure  $\geq 90$  mmHg, or self-report of high blood pressure) [36], peripheral artery disease (PAD), peripheral vascular operation (PVO), smoking status, therapy with statins and angiotensin-converting enzyme (ACE) inhibitors, and serum creatinine concentrations. Estimated glomerular filtration rate (eGFR) was calculated using the simplified modification of diet in renal disease formula ( $186.3 \times \text{serum creatinine}^{-1.154} \times \text{age}^{-0.203}$  ( $\times 0.742$  if female) ( $\times 1.212$  if black)) [37]. Plasma homocysteine and c-reactive protein (CRP) were determined by reversed-phase high performance liquid chromatography (HPLC) coupled to a fluorescence detector (ImmuChrom

GmbH, Heppenheim, Germany) and BN ProSpec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany), respectively, as previously described [38]. The demographic characteristics of the patients under study are summarized in Table 1.

#### Determination of plasma OPG and RANKL concentrations by enzyme-linked immunosorbent assay (ELISA)

Plasma OPG and RANKL concentrations were measured by solid-phase ELISA using commercially available kits purchased from R&D Systems Europe, Ltd, UK. The assays of OPG and RANKL are capable of determining total OPG, including monomer, dimer and bound form, and uncomplexed RANKL form, respectively. All procedures were performed according to the manufacturers' protocols and samples were diluted 20-fold. Every sample was run in duplicate, measurements differed by less than 10%, and the mean value was calculated and used for statistical analysis. A standard curve was created, and OPG and RANKL concentrations of the examined samples multiplied by the dilution factor was calculated and expressed in pg/ml. The sensitivity of the OPG assay was 1.4 pg/ml and the intra- and inter-assay precision coefficient of variation ranged between 3.2–4.6% and 5.5–7.7%, respectively, at different levels. The sensitivity of the RANKL assay was 2.1 pg/ml and the intra- and inter-assay precision coefficient of variation ranged between 3.6–4.9% and 6.1–8.2%, respectively, at different levels.

#### Statistical analysis

The Kolmogorov-Smirnov test was initially applied to assess the normality of distribution of plasma OPG and RANKL concentrations. Plasma OPG concentrations were normally distributed, therefore Student's t-test analysis was used to evaluate its association with categorical clinical variables. Plasma RANKL concentrations were not normally distributed and therefore the non-parametric method, Mann-Whitney U-test, was applied to assess its association with categorical clinical variables. Results were presented as mean (Standard Deviation-SD) and median (interquartile range-IQR: Q25-Q75) values for plasma OPG and RANKL concentrations, respectively. Spearman rank order correlation analysis was used to evaluate the linear relationship of OPG and RANKL concentration with continuous clinical variables. Multiple regression analysis was performed to assess which clinical variables are independent predictors of OPG and RANKL concentrations. A 2-tailed exact p-value of less than 0.05 was taken to be statistically significant. Statistical analyses were performed using SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL, USA).

## RESULTS

#### Clinical evaluation of plasma OPG concentrations

Plasma OPG concentrations ranged from 40.11 to 361.32 pg/ml with a mean value (SD) of 141.07 pg/ml ( $\pm 57.02$  pg/ml). Kolmogorov-Smirnov testing showed normal distribution for plasma OPG concentrations (K-S  $d=0.066$ ,  $p>0.05$ ). Student's t-test analysis was therefore applied to evaluate the associations between OPG concentrations and patients' clinical variables (Table 1).

When categorizing by the median age, plasma OPG concentrations were significantly increased in patients over 72 years compared to those under 72 years (Table 1,  $150.77\pm 57.49$  vs.  $128.86\pm 54.47$  pg/ml,  $p=0.0283$ ). Spearman rank order correlation analysis further indicated a positive association between plasma OPG concentrations and patients' age ( $R_s=0.1946$ ,  $p=0.0258$ ). Significant associations of OPG concentrations with homocysteine ( $R_s=0.3979$ ,  $p<0.00001$ ) and creatinine ( $R_s=0.1777$ ,  $p=0.0455$ ) levels, as well as eGFR ( $R_s=-0.1983$ ,  $p=0.0254$ ) were noted. OPG concentrations also showed a trend of correlation with CRP, without reaching statistical significance ( $R_s=-0.1472$ ,  $p=0.0998$ ). Diabetic patients presented significantly increased plasma OPG concentrations compared to non-diabetics (Table 1,  $157.38\pm 53.39$  vs.  $134.15\pm 57.37$ ,  $p=0.0324$ ). Plasma OPG concentrations were significantly increased in patients receiving therapy with statins ( $161.13\pm 50.71$  vs.  $131.26\pm 57.61$  pg/ml,  $p=0.0044$ ). Patients with history of hyperlipidemia showed significantly reduced OPG concentrations compared to non-hyperlipidemic patients ( $134.92\pm 58.22$  vs.  $157.91\pm 50.60$  pg/ml,  $p=0.0407$ ). Patients with history of smoking presented significantly reduced OPG levels compared to non-smokers ( $132.32\pm 46.99$  vs.  $155.69\pm 68.77$  pg/ml,  $p=0.0226$ ). Patients with history of CAD also showed significantly reduced OPG levels ( $130.17\pm 47.37$  vs.  $150.85\pm 63.22$  pg/ml,  $p=0.0377$ ). OPG concentrations were not associated with patients' sex, carotid position, stenosis grade, history of symptoms, hypertension, CABG/PTCA, PAD and PVO and therapy with ACEs (Table 1,  $p>0.05$ ). OPG concentrations were significantly increased in patients with amaurosis fugax compared to asymptomatic patients ( $168.41\pm 66.32$  vs.  $128.79\pm 45.28$  pg/ml,  $p=0.0284$ ).

Multiple regression analysis showed that history of diabetes ( $B=0.1940$ ,  $CI=0.0372-0.3507$ ,  $p=0.0157$ ), CAD ( $B=0.2487$ ,  $CI=0.0858-0.4116$ ,  $p=0.0030$ ), smoking status ( $B=0.1786$ ,  $CI=0.0229-0.3343$ ,  $p=0.0249$ ), therapy with statins ( $B=0.2480$ ,  $CI=0.0775-0.4185$ ,  $p=0.0047$ ) and homocysteine levels ( $B=0.2155$ ,  $CI=0.0594-0.3715$ ,  $p=0.0072$ ) were independent predictor factors of plasma OPG concentrations. In contrast, patients' age, history of hyperlipidemia and eGFR (or creatinine concentrations if used instead of eGFR) were not found to exert a significant independent effect on OPG concentrations ( $p>0.05$ ).

#### Clinical evaluation of plasma RANKL concentrations

Plasma RANKL concentrations ranged from 13.10 to 651.77 pg/ml, with a median value (IQR) of 117.43 pg/ml ( $37.53-75.77$  pg/ml). Kolmogorov-Smirnov test showed that plasma RANKL concentrations were not normally distributed (K-S  $d=0.337$ ,  $p<0.01$ ). The non-parametric method, Mann-Whitney U-test, was therefore used to assess the associations between plasma RANKL concentrations and patients' clinicopathological variables (Table 1).

When categorizing by the median age, plasma RANKL concentrations were significantly increased in patients over 72 years compared to those under 72 years [Table 1,  $130.71$  ( $81.14-405.81$ ) vs.  $89.73$  ( $28.01-133.77$ ) pg/ml,  $p=0.0114$ ]. Spearman rank order correlation analysis also revealed a positive association between plasma RANKL concentrations and patients' age ( $R_s=0.2045$ ,  $p=0.0191$ ). Plasma RANKL concentrations were significantly associated with plasma

**Table 1.** Associations of plasma OPG and RANKL concentrations with medical history, risk factors and medication intake in 131 patients with advanced carotid atherosclerotic lesions.

| Clinicopathological variables | N   | OPG (pg/ml)        |         | RANKL (pg/ml)          |         |
|-------------------------------|-----|--------------------|---------|------------------------|---------|
|                               |     | Mean $\pm$ SD      | p-value | Median (IQR)           | p-value |
| Age                           |     |                    | 0.0283  |                        | 0.0114  |
| <72                           | 58  | 128.86 $\pm$ 54.47 |         | 89.73 (28.01–133.77)   |         |
| $\geq$ 72                     | 73  | 150.77 $\pm$ 57.49 |         | 130.71 (81.14–405.81)  |         |
| Gender                        |     |                    | 0.4393  |                        | 0.3810  |
| Male                          | 105 | 139.14 $\pm$ 52.98 |         | 114.37 (35.80–343.68)  |         |
| Female                        | 26  | 148.85 $\pm$ 71.74 |         | 139.38 (72.50–400.50)  |         |
| Carotid                       |     |                    | 0.7385  |                        | 0.8847  |
| Right                         | 71  | 142.61 $\pm$ 56.25 |         | 116.41 (35.80–357.60)  |         |
| Left                          | 60  | 139.25 $\pm$ 58.33 |         | 117.43 (45.81–386.60)  |         |
| Stenosis grade                |     |                    | 0.8950  |                        | 0.6772  |
| <90%                          | 70  | 141.68 $\pm$ 61.95 |         | 117.64 (35.80–378.40)  |         |
| $\geq$ 90%                    | 61  | 140.36 $\pm$ 51.26 |         | 109.26 (46.46–319.70)  |         |
| Symptoms                      |     |                    | 0.6726  |                        | 0.7255  |
| No                            | 67  | 139.00 $\pm$ 56.29 |         | 117.43 (49.27–321.89)  |         |
| Yes                           | 64  | 143.23 $\pm$ 58.12 |         | 113.35 (35.47–518.14)  |         |
| Diabetes                      |     |                    | 0.0324  |                        | 0.1116  |
| No                            | 92  | 134.15 $\pm$ 57.37 |         | 110.28 (29.20–364.90)  |         |
| Yes                           | 39  | 157.38 $\pm$ 53.39 |         | 123.56 (85.50–484.39)  |         |
| Hyperlipidaemia               |     |                    | 0.0407  |                        | 0.9236  |
| No                            | 35  | 157.91 $\pm$ 50.60 |         | 117.43 (44.89–321.89)  |         |
| Yes                           | 96  | 134.92 $\pm$ 58.22 |         | 116.92 (34.94–386.60)  |         |
| Hypertension                  |     |                    | 0.6714  |                        | 0.3272  |
| No                            | 30  | 144.96 $\pm$ 68.77 |         | 113.34 (29.34–133.77)  |         |
| Yes                           | 101 | 139.91 $\pm$ 53.37 |         | 117.86 (46.46–384.20)  |         |
| Smoking status                |     |                    | 0.0226  |                        | 0.0185  |
| No                            | 48  | 155.69 $\pm$ 68.77 |         | 130.71 (72.50–578.96)  |         |
| Yes                           | 83  | 132.32 $\pm$ 46.99 |         | 107.73 (29.07–287.42)  |         |
| Statins                       |     |                    | 0.0044  |                        | 0.0004  |
| No                            | 88  | 131.26 $\pm$ 57.61 |         | 93.80 (24.31–300.41)   |         |
| Yes                           | 43  | 161.13 $\pm$ 50.71 |         | 132.75 (112.33–501.22) |         |
| ACES                          |     |                    | 0.6508  |                        | 0.3861  |
| No                            | 69  | 143.21 $\pm$ 61.95 |         | 112.23 (29.34–343.10)  |         |
| Yes                           | 62  | 138.67 $\pm$ 51.37 |         | 119.87 (71.58–378.40)  |         |
| CAD                           |     |                    | 0.0377  |                        | 0.3193  |
| No                            | 69  | 150.85 $\pm$ 63.22 |         | 114.37 (32.77–287.42)  |         |
| Yes                           | 62  | 130.17 $\pm$ 47.37 |         | 117.43 (74.59–400.50)  |         |

**Table 1 continued.** Associations of plasma OPG and RANKL concentrations with medical history, risk factors and medication intake in 131 patients with advanced carotid atherosclerotic lesions.

| Clinicopathological variables | N   | OPG (pg/ml)        |         | RANKL (pg/ml)         |         |
|-------------------------------|-----|--------------------|---------|-----------------------|---------|
|                               |     | Mean $\pm$ SD      | p-value | Median (IQR)          | p-value |
| CABG/PTCA                     |     |                    | 0.2502  |                       | 0.2497  |
| No                            | 93  | 144.74 $\pm$ 61.28 |         | 117.43 (32.77–313.40) |         |
| Yes                           | 38  | 132.07 $\pm$ 44.35 |         | 115.90 (81.14–508.63) |         |
| PAD                           |     |                    | 0.1004  |                       | 0.0577  |
| No                            | 96  | 136.12 $\pm$ 54.23 |         | 118.81 (29.20–316.55) |         |
| Yes                           | 35  | 154.62 $\pm$ 62.88 |         | 132.20 (88.92–505.63) |         |
| PVO                           |     |                    | 0.9933  |                       | 0.4646  |
| No                            | 122 | 141.05 $\pm$ 57.70 |         | 115.90 (35.80–343.63) |         |
| Yes                           | 9   | 141.22 $\pm$ 49.71 |         | 198.09 (95.70–384.20) |         |

homocysteine ( $R_s=0.3546$ ,  $p<0.00001$ ). Plasma RANKL concentrations showed a trend of correlation with eGFR ( $R_s=-0.1710$ ,  $p=0.0545$ ), whereas non-associations with serum creatinine ( $R_s=0.1263$ ,  $p=0.1365$ ) and CRP ( $R_s=-0.0811$ ,  $p=0.3665$ ) concentrations were noted. Patients with history of smoking showed significantly reduced RANKL levels compared to non-smokers [107.73(29.07–287.42) *vs.* 130.71 (72.50–978.96) pg/ml,  $p=0.0185$ ]. RANKL concentrations were significantly increased in patients receiving therapy with statins compared to untreated patients (130.71(72.50–578.96) *vs.* 107.73(29.07–287.42) pg/ml,  $p=0.0004$ ). Patients with history of PAD also showed increased RANKL levels compared to those with no evidence of PAD, without reaching statistical significance [132.20 (88.92–605.63) *vs.* 118.81 (29.20–316.55) pg/ml,  $p=0.0577$ ]. RANKL concentrations were not associated with patients' sex, carotid position, stenosis grade, history of symptoms, diabetes, hypertension, hyperlipidemia, CAD, CABG/PTCA and PVO and therapy with ACEs (Table 1). RANKL concentrations showed a strong positive correlation with OPG concentrations ( $R_s=0.3356$ ,  $p<0.00001$ ).

Multiple regression analysis showed that smoking status ( $B=0.2952$ ,  $CI=0.1215-0.4689$ ,  $p=0.0010$ ) was an independent predictor of plasma RANKL concentrations. In contrast, patients' age, therapy with statins and homocysteine concentrations were not found to exert a significant independent effect on RANKL concentrations ( $p>0.05$ ).

## DISCUSSION

In the last few years, a gradually increasing number of animal and human studies have been performed in order to assess the potential role of the OPG/RANKL/RANK axis in vascularity. The most comprehensive data from existing clinical studies reported an association between elevated OPG and/or RANKL levels and the presence, severity and progression of cardiovascular diseases, including carotid atherosclerosis [30–34].

In the present study, plasma OPG levels were increased in elderly and diabetic patients with advanced carotid

atherosclerotic lesions, who underwent carotid endarterectomy for vascular repair. These findings are consistent with previous clinical studies conducted on different study populations [26–29]. RANKL levels were associated with patients' age, presenting only a trend of correlation with history of diabetes. OPG and RANKL concentrations also showed a positive association with homocysteine levels, a well-established risk factor for cardiovascular disease [39]. On the other hand, we did not find any statistical difference in plasma OPG levels between symptomatic and asymptomatic patients with carotid artery stenosis, which is in contrast with the findings of previous studies [30,32]. However, OPG concentrations were significantly increased in patients with amaurosis fugax compared to asymptomatic patients. In this aspect, recent epidemiological studies have indicated age- and sex-specific actions of OPG in carotid atherosclerosis progression, providing a possible explanation for the above controversy [40,41]. We also showed that increased OPG and RANKL levels were associated with history of PAD, but without reaching statistical significance. In this aspect, Golledge et al recently reported an association between elevated serum OPG levels and impaired endothelium, measured as decreased flow-mediated dilatation of the brachial artery in patients with peripheral artery disease [42]. Patients with clinical stage III or IV PAD also presented increased plasma OPG concentrations in comparison to those without ischemic ulcerations [43,44].

The present study further documented that OPG, but not RANKL levels, were reduced in patients with history of CAD and hyperlipidemia, but with the latter not exerting an independent effect in multiple regression analysis. Both OPG and RANKL levels were also significantly reduced in patients with history of smoking compared to non-smokers. In this aspect, the most comprehensive data to date highlighted the ability of circulating OPG levels to predict the prevalence and severity of CAD [45–48]. Plasma OPG levels were increased in patients with acute coronary syndrome compared to those with stable angina or normal coronary arteries, being associated with CAD progression [45–48]. However, the inverse correlation between OPG levels and history of CAD found in the present study is not directly

opposed to previous data, since the influence of CAD in OPG levels may be complicated by the multiple risk factors governing advanced carotid atherosclerosis of the current study population. Such controversies have also been reported in several clinical studies conducted on general populations for both OPG and RANKL [49–51]. Moreover, OPG effects may differ depending on the stage of atherosclerotic lesion. In early stages OPG may be increased in order to protect vessels by activating inflammatory pathways in an effort to compensate vasculature damage [26–29]. As atherosclerotic lesion progresses, OPG may become injurious to the vessels or is just unable to reverse the procedure of vascular calcification [26–29].

We also found that patients receiving therapy with statins exhibited significantly increased OPG and RANKL levels compared to untreated patients. Statins have been shown to exert many favorable effects, including normalizing atherogenic lipid profile, reduction of inflammation, improvement of endothelial function and decrease of cardiovascular morbidity and mortality [52]. Notably, several recent studies have investigated the effects of statins therapy on circulating OPG and RANKL levels. However, the existing studies have been performed on different cohorts, including patients with diabetes type 2 only or CAD only, as well as patients with both diabetes type 2 and microalbuminuria or hypercholesterolemia [53–56]. Among them, 3 studies documented serum OPG elevation during statins therapy (lovastatin or simvastatin therapy) [53,54,56], whereas another study reported OPG reduction during pravastatin therapy [55]. Serum RANKL levels were only assessed in 1 of the above studies, being reduced during lovastatin therapy [55]. It should be noted that the majority of the current study population received therapy with a different statin, atorvastatin (27 out of 43 patients receiving therapy with statins), whereas a smaller proportion of patients received simvastatin, fluvastatin or rosuvastatin therapy (5, 6 and 7 patients, respectively). In this aspect, the existing discrepancies may be ascribed to differences in type of statin, administered dosage and study population, reinforcing the need for further research in order to determine the molecular basis of statins' effects on OPG and RANKL levels.

Plasma OPG concentrations were also associated with serum creatinine levels and eGFR, which may be ascribed to the fact that patients with advanced carotid atherosclerosis usually exhibit renal impairment. A trend of correlation between RANKL and eGFR was also noted, but without reaching statistical significance. However, it should also be noted that neither OPG nor RANKL levels were associated with history of hypertension, which may induce renal injury, especially in patients with multiple risk factors. In this context, OPG levels were shown to be increased in predialysis and dialysis patients with chronic kidney disease, being associated with the presence and severity of aortic and coronary calcification [57–59]. Notably, taking into consideration that serum OPG levels were reduced within 14 days of renal transplantation, it was supported that OPG elevation in chronic kidney disease patients could be ascribed to its accumulation due to impaired renal clearance [60].

In general, it should be taken into account that the cohorts of the existing clinical studies exhibited considerable variations, as some investigations were restricted to patients with

diabetes or CAD, while others were conducted on healthy populations, rendering the information concerning the participants considerably different and not directly comparable [26–29]. Furthermore, OPG and RANKL measurements were performed with many different kits, and assays were constructed with the use of different antibodies and calibrators. Many studies were based on serum OPG measurements, while others measured plasma OPG. As OPG was shown to behave differentially in those 2 matrices, the existing results cannot be directly compared [61]. Pre-analytical issues affecting the OPG concentrations in biological samples, such as enzymatic degradation, freeze-thaw and binding to other proteins, still need thorough investigation to assure correct and comparable measurements [26]. These population and analytical issues provide further possible explanations for the controversies amongst the existing clinical data and the present report. Furthermore, the limited clinical value of RANKL found in previous clinical studies and the present report may be ascribed to the fact that the existing assays exclusively measure the levels of uncomplexed RANKL, but not of RANKL that is bound to its decoy receptor OPG. On the other hand, the assays of OPG can determine total OPG, including monomer, dimer and bound form.

## CONCLUSIONS

In conclusion, the associations of plasma OPG concentrations with medical history, risk factors and medication intake supported evidence for the potential clinical implication of OPG in carotid atherosclerosis, whereas the clinical utility of RANKL seems uncertain. However, it remains unclear whether OPG and/or RANKL play a primary or a secondary causal role in mediating or protecting against vascular injury or are only markers of atherosclerosis progression. Further studies are recommended in order to elucidate the clinical significance of OPG and RANKL levels in cardiovascular pathologies, including carotid atherosclerosis, and to determine if functions related with OPG or its ligand may be targets for future therapeutic interventions.

## Conflict of interest statement

All authors verify that they have not accepted any funding or support from an organization that may in any way gain or lose financially from the results of the present study. All authors verify that they have not been employed by an organization that may in any way gain or lose financially from the results of the present study. None of the authors have any other conflicting interest.

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